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Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor.

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Running Title: Interaction of Rab11a with the Prostacyclin Receptor

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ABSTRACT
The cellular trafficking of numerous G protein-coupled receptors (GPCRs) is known to be regulated by Rab proteins that involves a direct protein:protein interaction between the receptor and the GTPase. In the case of the human prostacyclin receptor (hIP), it undergoes agonist-induced internalization and subsequent Rab11a-dependent recyclization involving an interaction between a Rab11-binding domain (RBD) localized within its carboxyl-tail domain with Rab11a. However, the GPCR-interacting domain on Rab11a itself is unknown. Hence, we sought to identify the region within Rab11a that mediates its interaction with the RBD of the hIP. The α4 helix region of Rab11 was identified as a novel binding domain for the hIP, a site entirely distinct from the Switch I/Switch II -regions that act as specific binding domain for most other Rab and Ras-like GTPase interactants. Specifically, Glu¹³⁸ within α4 helix of Rab11a appears to contact with key residues (e.g Lys³⁰⁴) within the RBD of the hIP, where such contacts differ depending on the agonist-activated versus -inactive status of the hIP. Through mutational studies, supported by in silico homology modelling of the inactive and active hIP:Rab11a complexes, a mechanism is proposed to explain both the constitutive and agonist-induced binding of Rab11a to regulate intracellular trafficking of the hIP. Collectively, these studies are not only the first to identify α4 helix of Rab11a as a protein binding domain on the GTPase but also reveal novel mechanistic insights into the intracellular trafficking of the hIP, and potentially of other members of the GPCR superfamily, involving Rab11-dependent mechanisms.
1 INTRODUCTION

The prostanoid prostacyclin, or prostaglandin (PG) I\(_2\), plays an essential role within the cardiovascular system where it dynamically regulates platelet activation status and blood vessel tone [1]. It also acts as a cytoprotective agent during acute myocardial ischemia and enhances endothelial cell survival supporting neovascularisation and vascular repair in response to vessel injury [1-3]. Clinically, prostacyclin analogues are used in treating pulmonary arterial hypertension/PAH, a disease characterized by ongoing endothelial dysfunction and vascular remodelling ultimately resulting in right ventricular hypertrophy or heart failure [4]. Prostacyclin mainly signals through activation of the prostacyclin receptor which, according to IUPHAR nomenclature is known as the I prostanol receptor or, in short, as the IP, a member of the G protein coupled receptor (GPCR) superfamily primarily coupling to Gs/adenylyl cyclase activation [5]. The IP is unusual among GPCRs in that it undergoes both isoprenylation and palmitoylation within its carboxyl-terminal (C-tail) domain, post-translational lipid modifications essential for IP signalling and function [5, 6]. Specifically, in the case of the human (h) IP, it is farnesylated at Cys\(^{383}\) within an evolutionary conserved -CAAX motif [5], and is palmitoylated at multiple Cys residues (Cys\(^{308}\), Cys\(^{309}\) and Cys\(^{311}\)) within its proximal C-tail domain [6]. These dual lipidations introduce a double loop structure within the C-tail domain of the hIP to orientate and provide the binding domain(s) for interaction with its coupling G protein(s) and with components of the protein trafficking machinery [6]. In addition, the hIP contains a novel endoplasmic reticulum (ER) export motif and can be subject to 26S proteasomal or lysosomal degradation depending on the processing- and maturation-status of the receptor [7, 8].

As a member of the GPCR superfamily, a critical factor affecting the ability of the IP to modulate prostacyclin signalling is its tendency to undergo agonist-induced desensitization involving receptor phosphorylation and/or intracellular- trafficking [9]. In general, desensitization of GPCRs serves to sequester the receptor from the plasma membrane through internalization followed by recycling back to the membrane for further signalling or targeting it to the lysosomes for degradation [9]. In the case of the hIP, it undergoes agonist-induced internalization through a Rab5a-dependent mechanism [10] with subsequent recycling on Rab11-positive slow-recycling endosomes found to occur through a mechanism involving a direct interaction between the C-tail domain of the hIP with Rab11a [11]. Through detailed localization studies, the Rab11-binding domain (RBD) within the hIP required for its specific interaction with Rab11a was localised to hIP\(^{299-312}\), a region of the hIP comprising its eighth \(\alpha\)-helical domain (\(\alpha\)-H8), corresponding to Val\(^{309}\)-Val\(^{307}\), and lying adjacent to the palmitoylated residues at Cys\(^{308}\) -Cys\(^{311}\) [6]. Ala-scanning mutagenesis of the RBD of the hIP identified several key residues within the \(\alpha\)-H8 domain, namely F300, R302, L303, K304, L305, V307, as essential for its interaction with Rab11a [6]. Furthermore, through additional mechanistic studies, it was determined that constitutive palmitoylation at Cys\(^{311}\), along with agonist-regulated deacylation (removal of the palmitate) of Cys\(^{308}\) and Cys\(^{309}\), may orientate and position the essential residues of the \(\alpha\)-H8/RBD in proximity to Rab11a to facilitate interaction of the hIP with Rab11a [6].

Similar to the hIP, there is a growing list of GPCRs now established to directly associate with Rab11a. These include the angiotensin II type 1A receptor (AT\(_1\)aR), the beta-2 adrenergic receptor (\(\beta\)_2AR), and the TP\(\beta\) isoform of the human thromboxane (TX) A\(_2\) receptor [12-14]. The RBDs within each of these GPCRs have been characterised and, similar to that of the hIP, are all localised within their C-tail domains. However, and also similar to that of the hIP, the actual region(s) within Rab11a itself involved in these GPCR interactions have not yet been identified.

Rab11a is a member of the Rab11 subfamily of Rab GTPases, which comprises two other members, namely Rab11b and Rab25 [15]. High-resolution X-ray crystallography structures for Rab11a show that it adopts an overall protein fold common to all Ras-like GTPases, including other members of the wider Rab
protein family [16, 17]. As depicted in **Figure 1A**, this GTPase fold comprises a six-stranded β-sheet core (β1-β6), surrounded by five α-helices (α1-α5), with the loop regions connecting the β-sheets and α-helices being responsible for guanine nucleotide-binding and GTP hydrolysis [18, 19]. Of note, due to its sequence divergence and/or multiple possible conformers, the majority of the elucidated structures of the Rab GTPases do not include the carboxyl-terminal hypervariable domain (HVD). It is postulated that the HVD acts as a signal for targeting the particular Rab protein to its specific intracellular compartment(s) [20-22].

While the overall structure of the Rab protein family is highly conserved, it is the unique conformation that each active/GTP-bound Rab structure adopts that determines its specific function, such as through interaction with specific protein effectors. Like other Ras-like GTPases, Rab proteins contain two domains, termed **Switch I** and **Switch II**, that make contact with the γ-phosphate of GTP, and it is within these regions that the most significant conformational changes occur between the inactive/GDP-bound and the active/GTP-bound states [23, 24]. In turn, the active/GTP-bound Rab protein interacts with numerous specific effector proteins to mediate its given function [19, 25]. Among the known effector interactants of Rab11a, the “family of Rab11 interacting proteins” (FIPs) are the best characterised [26]. The FIPs comprise five members, namely FIP1, also referred to as Rab-coupling protein (RCP), FIP2, FIP3, FIP4 (Arfophilin-2), and FIP5 (Rip11) [27-30]. Crystallographic structural determination of Rab11a in complex with FIP2 and FIP3 show that the binding interfaces within Rab11a mediating both interactions are mainly restricted to the **Switch I** (residues 39-46) and **Switch II** (residues 68-79) regions [31, 32]. These findings are indicative of the conformational changes present solely within the GTP-bound Rab11a which are required to mediate the recruitment of, and interaction with, its key effector proteins.

Thus, while the region of interaction between the Rab GTPases and its key interactants &/or effector proteins involves the **Switch II/Switch II** domains, to the best of our knowledge those specific regions or domains within Rab11a itself involved in its interaction with GPCRs have not actually been identified. Hence, the aim of this study was to further investigate the direct interaction between the hIP and Rab11a with the specific objective of identifying the region(s) within Rab11a that mediate its interaction with the RBD of the hIP. Our studies reveal a novel binding site for the hIP within Rab11a, corresponding to the α4 helix of Rab11a, and which is not only entirely distinct from **Switch II/Switch II** identified as the specific interacting domains for all other interactants of Rab11a but also for all other Ras-like GTPases [31, 32]. In addition, through a combination of experimental approaches along with in silico homology modelling of the inactive and active (agonist-bound) hIP, and of the hIP:Rab11a complex in both activation states, a mechanism is proposed to explain the binding of Rab11a with the hIP both constitutively, in the absence of agonist, and in response to agonist-induced receptor activation.
2 MATERIALS AND METHODS

2.1 Expression Plasmids

The plasmids pGBKT7:hiP299–386, pGBKT7:hiP299–386, SSLC, pGBKT7:hiP312–386, pGBKT7:hiP299–312 and pEGFP-C1:Rab11a have been previously described [6, 11]. The plasmids pACT2:Rab11a124–216, pACT2:Rab11a131–216, pACT2:Rab11a146–216, pACT2:Rab11a158–216 and pACT2:Rab11a174–216 were generated by subcloning the respective PCR-amplified Rab11a subfragments from pEGFP-C1:Rab11a into the 5’ Neol and 3’ EcoRI sites of pACT2, in-frame with the translation of the activation domain (AD) of the yeast GAL4 transcriptional activator (GAL4-AD) (See Supplemental Table 1 for respective oligonucleotide primer sequences). The plasmids pACT2:Rab11a124–210, pACT2:Rab11a124–197, pACT2:Rab11a124–187, pACT2:Rab11a124–216, T136A, pACT2:Rab11a124–216, D137A, pACT2:Rab11a124–216, E138A, pACT2:Rab11a124–216, R140A, pACT2:Rab11a124–216, F142A, pACT2:Rab11a124–216, E144A and pACT2:Rab11a124–216, K145A were generated by QuikChange site-directed mutagenesis (SDM) using pACT2:Rab11a124–216 as template (See Supplemental Table 2 for respective oligonucleotide primer sequences). The plasmids pEGFP-C1:Rab11aT136A, pEGFP-C1:Rab11aD137A, pEGFP-C1:Rab11aE138A, pEGFP-C1:Rab11aR140A, pEGFP-C1:Rab11aF142A, pEGFP-C1:Rab11aE144A and pEGFP-C1:Rab11aK145A were generated by QuikChange SDM using pEGFP-C1:Rab11a as template (See Supplemental Table 2 for respective oligonucleotide primer sequences). The bacterial expression plasmid pET-28b:Rab11a1–173 encoding a hexahistidine-tagged, carboxy-terminally truncated Rab11a has been previously described [32]. The plasmids pET-28b:Rab11a1–173, T136A, pET-28b:Rab11a1–173, D137A, pET-28b:Rab11a1–173, E138A, pET-28b:Rab11a1–173, R140A, pET-28b:Rab11a1–173, F142A, pET-28b:Rab11a1–173, E144A and pET-28b:Rab11a1–173, K145A were generated by QuikChange SDM using pET-28b:Rab11a1–173 as template (See Supplemental Table 2 for respective oligonucleotide primer sequences). The plasmid pET-28b:Rab11a1–173, E138A, E144A was generated by QuikChange SDM using pET-28b:Rab11a1–173, E144A as template (See Supplemental Table 2 for oligonucleotide primer sequences). All plasmids were validated by DNA sequence analysis (Eurofins MWG Operon; Ebersberg, Germany). The mammalian expression vectors encoding haemagglutinin (HA)-epitope tagged forms of the hIP (pHM6:hiP), hiPK304A (pHM6:hiP304A), hiPK304Q (pHM6:hiP304Q) and hiPK304R (pHM6:hiP304R) have been previously described [7].

2.2 Yeast Matings and Yeast Two-Hybrid Screening

Previous yeast-2-hybrid (Y2H) screening of a human kidney cDNA library with the carboxyl-terminal (C)-tail domain, encoding amino acids 299–386, of the hIP as bait protein identified Rab11a, expressed in the yeast prey plasmid pACT2:Rab11a, as a specific interactor of the hIP [11]. The plasmids pGBKT7 and pGBKT7:p53, encoding the GAL4 DBD alone or as a fusion with p53, were obtained from Clontech. All yeast protocols were standard procedures as previously described [11]. In brief, all pGBKT7-based bait plasmids were transformed into Saccharomyces cerevisiae (S.c) AH109 (MATa strain) while pACT2-based prey plasmids were transformed into S.c Y187 (MATa strain) and were mated with selection of diploids on synthetic double drop-out (DDO) media (SD/Leu, Trp). Positive interactions between bait and prey proteins were identified by expression of the HIS3, ADE2 and lacZ reporter genes on quadruple drop-out (QDO) media (SD/Leu, Trp, His, Ade) and for the ability to cleave Xβ-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), as measured by the filter lift assay of β-Galactosidase (β-Gal) activity. The scoring system used in the latter was based on the ability of 3 independent colonies selected from respective DDO media to produce blue (+) or white (-) colonies owing to expression of β-Gal activity, where ‘+++’ was used to indicate that cells developed blue colour within 30 min, ‘++’ was used to indicate that cells developed blue colour within 1 hr, ‘+’ was used to indicate that cells developed blue colour within 2 hr, and ‘-’ indicates cells remained white over the period of the assay (4 hr).
2.3 Cell Culture and Transfections
Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were cultured in minimal essential medium (MEM), supplemented with 10% (v/v) foetal bovine serum (FBS) and 2 mM L-glutamine. HEK.hIP and HEK.β-Gal cells, stably over-expressing haemagglutinin (HA)-tagged forms of the hIP and β-Gal respectively, have been previously described [5, 11]. Cells were transiently transfected using the Effectene transfection reagent (Qiagen), according to the manufacturer’s protocols. Transiently-transfected cells were harvested 48 hr post-transfection, unless otherwise stated. All mammalian cells were grown at 37 °C in a humid environment with 5% CO₂ and were routinely validated and checked to ensure they were free from mycoplasma contamination.

2.4 Immunoprecipitations
Association of the full length hIP and Rab11a proteins, or its Ala-scanning variants, was examined through co-immunoprecipitations from mammalian HEK 293 cell lines. HEK.hIP and HEK.β-Gal cells, stably over-expressing HA-tagged forms of the hIP and β-Gal, respectively, were transiently co-transfected with pEGFP-C1-based plasmids, encoding GFP-tagged forms of Rab11a (wild type/WT Rab11a, Rab11aT136A, Rab11aD137A, Rab11aE138A, Rab11aR140A, Rab11aF142A, Rab11aE144A and/or Rab11aK145A) together with pAdVA as indicated in the figure legends. Some 48 hr post-transfection, cells were washed in serum-free MEM and either incubated with vehicle or 1 µM cicaprost for 2 hr at 37 °C. Thereafter, incubations were stopped by washing the cells twice in ice-cold phosphate-buffered saline (PBS) followed by lysis in 600 µl radio-immunoprecipitation (RIP) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1mM EDTA, 1% (v/v) Nonidet P-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM sodium fluoride, 25 mM sodium pyrophosphate, 1 mM PMSF, 4 μg/ml leupeptin, 2.5 μg/ml aprotinin). Lysates were clarified by centrifugation at 13,000 g for 5 min and 50 μl (approximately 50 μg) was retained for analysis of protein contamination.

2.5 Rab11a Protein Expression
The plasmids pET-28b-Rab11a1-172 (6 × His) or the various Ala-scanning variants thereof, as detailed above, were transformed into E. coli BL21(DE3) cells and positive transformants were screened for strong protein expression using small-scale protein expression experiments. Positively-expressing isolates were inoculated into 20 ml of 2×YT medium (1.6% peptone, 1% yeast extract, 0.5% NaCl, supplemented with 50 μg/ml kanamycin) and grown overnight at 37 °C, with shaking at 250 rpm. The entire overnight cultures were transferred to 1 L of 2×YT medium, grown to log phase, induced with 1 mM IPTG and grown for an additional 4 hr. Cells were harvested by centrifugation (6000 g, 15 min, 4 °C) and pellets were re-suspended in 30 ml Extraction Buffer (10 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). The cell suspensions were homogenized using a piston to ensure that no clumps of cells remained, subjected to sonication (3 × 1 min, 30% duty) and the resultant lysates were clarified by centrifugation (16000 g, 45 min, 4 °C) to remove cellular debris. The supernatants were then loaded onto columns containing 2 ml Ni-NTA agarose resin (Qiagen) which were pre-equilibrated with 20 ml extraction buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). The column was then washed with 20 ml wash buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). Fractions containing the Rab11a protein were pooled and dialysed against buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). The Rab11a protein was then purified to homogeneity by passage through a 0.5 ml Ni-NTA column equilibrated with buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). The Rab11a protein was subjected to size-exclusion chromatography (HPLC) using a Superose 12 column equilibrated with buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0) and elution was monitored by absorbance at 280 nm. Fractions containing the Rab11a protein were pooled and concentrated using a Centricon 30 concentrator (Millipore).
buffer. After extensive washing with 50 ml wash buffer (10 mM Tris-Cl, 300 mM NaCl, 20 mM imidazole, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0), the bound proteins were eluted with elution buffer (10 mM Tris-Cl, 300 mM NaCl, 200 mM imidazole, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8.0). Fractions containing the eluted Rab11a proteins were pooled and dialysed overnight at 4 °C against low salt Dialysis Buffer (10 mM Tris-Cl, 10 mM NaCl, 1 mM DTT, pH 8.0) and protein concentrations were determined by Bradford assay. To confirm protein secondary structure, far-UV circular dichroism (CD) spectra were collected on a Jasco J810 spectropolarimeter. All samples were analysed in Dialysis Buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA pH 8.0) at a concentration of 4-8 μM, using a 1 mm cuvette at 25 °C. Scans were measured over 290 to 195 nm, at 20 nm/min, with response time of 2 sec, data pitch of 0.5 nm, bandwidth of 3 nm, and averaged over 5 accumulations. The spectra were smoothed using the Savitzky-Golay method in Spectra Analysis software (Jasco) with a convolution width of 15 nm, then converted to Mean Residue Ellipticity (MRE) values and overlaid to check for large deviations in secondary structure [33].

2.6 Surface Plasmon Resonance
A biotinylated IP peptide (pepIP296-312), corresponding to residues 296-312 encompassing the Rab-binding domain of the hIP, was synthesized by ThinkPeptides (Oxford, UK; 99.38% purity, HPLC). All surface plasmon resonance (SPR) experiments were performed at 25 °C using a Series S sensor chip SA with a BiaCore T200 SPR instrument (GE Healthcare). All experiments were performed in HBS-P Running Buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% (v/v) surfactant P20). The biotinylated IP peptide was diluted in Running Buffer and then immobilized to a density of 3000 response units (RU). The purified Rab11a proteins (at concentrations 1–4 μM, diluted in Running Buffer) or, as reference controls, equivalent volumes of Running Buffer only were then injected onto the chip surface for up to 30 min at a flow rate of 5 μl/min. The dissociation phases were monitored for up to 90 min. Individual sensorgrams were referenced against injection onto an empty flow cell and baseline binding level prior to protein injection was set to zero in all cases. Data were fitted to a 1:1 Langmuir model using Biaevaluate analysis software (GE Healthcare).

2.7 Computational Structure Prediction and Macromolecular Docking
The structural model for Rab11a was generated by online submission to the Iterative-Threading ASSEmbly Refinement (I-TASSER) service for protein structure and function prediction [34, 35]. For modelling of the full-length Rab11a protein to include its HVD, the amino acid sequence of Rab11a was threaded onto the Rab hypervariable domain-deficient high-resolution X-ray crystal structure of Rab11a-GDP (Protein Data Bank (PDB) accession code: 1OIV, [16]). For modelling, the structure for Rab11a in its GDP-bound state was chosen as it has been previously shown that its binding to the hIP occurs independently of the guanine nucleotide (GDP vs GTP)-binding status of Rab11a [11].

Homology models of the hIP in its inactive and active states were also generated using the I-TASSER algorithm [34, 35]. Specifically, to generate the proposed inactive state model of the hIP (Figure 4A, left panel), the full-length sequence of the hIP was threaded to the templates of the inverse agonist-occupied templates of the carazolol-bound β₃AR-T4L structure (PDB accession code: 2RH1, [36]) and the ZM241385-bound A₂AR (PDB accession code: 3EML, [37]). To generate the proposed active state model of the hIP (Figure 4A, right panel), the sequence was threaded to the agonist-occupied templates of the B167107-bound β₃AR (PDB accession code: 3P0G, [38]) and the NECA-bound adenosine A₂AR (PDB accession code: 2YDV, [39]). In general, the I-TASSER correlation score (C-score) for models generated using the algorithm ranges from -5 to 2, with higher scores representing increased confidences in the model. The C-scores for the highest ranked inactive and active models of the hIP were 0.3 and -0.5, respectively. To validate the homology models of the
hIP, the structures were submitted to PROCHECK [40], wherein it was confirmed that the majority (> 91%) of the main chain conformations of both models were located in favoured regions of the Ramachandran plot (data not shown). Of the remaining residues, some 8% were found in acceptable regions, while only 1% were found in disallowed regions. Residues in the disallowed regions were primarily located within the distal C-tail domain, where the quality of the modelling would be expected to be low due to the truncation of this region within the template GPCR structures used to generate the homology models of the hIP.

All structural alignments and structural figures were generated using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.). Macromolecular docking simulations of the models of the hIP and Rab11a were performed using the HADDOCK (High Ambiguity Driven DOCKing) package [41], which allows for direct incorporation of experimental findings as restraints to drive the docking algorithm.

2.8 Statistical Analysis
Statistical analyses of differences were analysed by two-tailed unpaired Student’s t-test or one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison post hoc test, as indicated, using GraphPad Prism, version 6.0. All values are expressed as mean ± standard error of the mean (SEM). p values of < 0.05 were considered to indicate a statistically significant difference. As relevant, single (*), double (**), and triple (***), asterisk symbols signify p < 0.05, < 0.01, and < 0.001, respectively.
3 RESULTS

3.1 Identification of the domain(s) of Rab11a involved in mediating its interaction with the hIP

Rab11a has been identified as a direct functionally important interactant of the hIP, which occurs through an association involving a Rab11-binding domain (RBD) localized within the C-tail domain of the hIP [6, 11]. However, the specific region or domain within Rab11a itself that mediates its interaction with the RBD of the hIP is unknown and remains to be identified. The interaction of the hIP with Rab11a was initially identified through a Y2H screen of a human kidney cDNA library where the C-tail domain of the hIP (hIP[299-386]) was used as the specific bait protein [11]. This Y2H screen identified several independent clones of Rab11a, where each encoded residues 124-216 corresponding to the C-terminal region of Rab11a. A homology model of the structure of full-length Rab11a including its hypervariable domain (HVD) was generated by using the I-TASSER algorithm (Figure 1A, upper panel). From the homology model, it is evident that the region encoded by Rab11a[124-216], identified in the Y2H screens, encompasses the structural elements α4, β6 and α5, along with the entire HVD (Figure 1A, lower panels).

To facilitate the identification of the domains or regions of Rab11a[124-216] critical for its interaction with the hIP, a range of N (amino)-terminal truncated prey proteins were generated from Rab11a[124-216] and examined in Y2H screens with various bait proteins corresponding to subfragments of the C-tail domain derived from either the wild-type (hIP[299-386,WT]) or the isoprenylation-defective (hIP[299-386,SLC]) forms of the hIP (Figure 1B). In agreement with previous studies [6, 11], Rab11a[124-216] only interacted with hIP[299-386,WT] and hIP[299-312] subfragments, each of which contained the putative RBD of the hIP (Figure 1B). Notably, the hIP[299-312] subfragment containing only the RBD (α-H8 and adjacent Cys308-311 residues) but devoid of the rest of the C-tail domain of the hIP was sufficient to support the interaction with the Rab11a prey protein. Conversely, Rab11a[124-216] did not interact with the hIP[312-386] subfragment or with the control bait strains, encoding p53 or the empty vector (Ø) alone (Figure 1B). Furthermore, as additional controls, only p53 interacted with the SV40 large T-antigen, encoded by the plasmid pTD1-1, while there was no interaction between any of the bait proteins and the GAL4-AD, encoded by the empty prey vector pACT2 (Supplemental Figure 1). Similar to Rab11a[124-216], Rab11a[131-216], which lacks part of the loop region (residues 124-130) immediately preceding the α4 helix, interacted with all hIP baits containing the RBD. In contrast, Rab11a[146-216] devoid of the α4 helix (residues 136-145) failed to interact with any of the hIP subfragments, including those containing the RBD (Figure 1B). Consequently, further N-terminally truncated prey proteins, namely Rab11a[158-216] and Rab11a[174-216], devoid of the β6 strand and α5 helix, respectively, also failed to show an interaction with the subfragments of the hIP tested (Figure 1B).

Thereafter, to establish if other regions of the Rab11a[124-216] prey may also be involved in mediating the hIP:Rab11a interaction, such as within the HVD, a range of carboxyl (C)-terminal truncated prey proteins were generated and tested for interaction with the hIP-based subfragments through Y2H screening (Supplemental Figure 2). Deletion of the immediate C-terminal region in Rab11a[124-210], removing the membrane association domain (MAD), or of extended regions of the HVD in Rab11a[124-197] and Rab11a[124-187] did not affect their ability to interact with the hIP[299-386,WT]/hIP[299-312] or hIP[299-312] baits containing the RBD (Supplemental Figure 2).

Collectively these data, generated using the Y2H-based screening approach, suggest an essential requirement for the α4 helix of Rab11a, spanning residues 136-145, in mediating its interaction with the RBD of the hIP.

3.2 Assessment of the hIP:Rab11a interaction through Ala-scanning mutagenesis of α4 helix of Rab11a
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

Thereafter, Ala-scanning mutagenesis was used to interrogate the role of individual residues within the α4 helix of Rab11a required for its interaction with the hIP, initially using Y2H-based studies. In all cases, computational secondary structure prediction, using Jpred [42], confirmed that introduction of the mutations per se did not affect formation of the α4 helix within the variant Rab11a proteins. Mutation of Glu138 (E138A) and Glu144 (E144A) within Rab11a124-216 either abolished or impaired its interaction with the hIP299-386, WT/SSLc or hIP299-312 subfragments, respectively (Figure 2A). Furthermore, mutation of Lys145 (K145A) impaired the interaction of Rab11a124-216 with the RBD-containing baits (hIP299-386, WT/SSLc and hIP299-312), while mutation of the residues Thr136, Asp137, Arg140 and Phe142 had no effect (Figure 2A).

The association between the full length hIP and Rab11a proteins expressed in mammalian cells was investigated through co-immunoprecipitation studies carried out both in the absence (constitutive) and presence of the selective IP agonist cicaprost [43]. To this end, HEK.hIP cells [5] and, as controls, HEK.β-Gal cells encoding HA-tagged forms of the hIP and β-Gal, respectively, were transfected with expression vectors encoding GFP-tagged Rab11a, and the presence of Rab11a in the respective anti-HA immunoprecipitates examined. Consistent with previous reports [11], Rab11a showed a constitutive interaction with the hIP and was detected in the anti-HA immunoprecipitates from HEK.hIP cells in the absence of agonist (Figure 2B, upper panels, & 2C). Following stimulation with cicaprost for 2 hr, there was a significant agonist-dependent increase in the level of Rab11a associated with the hIP immunoprecipitate (Figure 2B, upper panels, & 2C).

Furthermore, Ala-scanning mutagenesis of the full-length Rab11a within its α4 helix domain (Rab11aT136A, Rab11aD137A, Rab11aE138A, Rab11aE144A, Rab11aR140A, Rab11aF142A, Rab11aE144A and/or Rab11aK145A) and expression in mammalian HEK.hIP cells allowed for assessment of the individual residues of the α4 helix of Rab11a in the agonist-independent/constitutive and agonist-dependent interactions with the hIP. In the absence of agonist, each of the Rab11a variants were present at similar levels to that of the wild type Rab11a in the anti-HA immunoprecipitates from HEK.hIP cells suggesting that they can all constitutively associate with the hIP (Figure 2B, upper panels & Supplemental Figure 3, left upper panels). However, mutation of Asp137 (D137A), Glu138 (E138A), Glu144 (E144A) and Lys145 (K145A) each significantly disrupted the agonist-induced increase in the interaction of Rab11a with the hIP, where the greatest impact was observed on mutation of E138A > E144A > K145A > D137A (Figure 2B, upper panels, Figure 2C & Supplemental Figure 3, right upper panels). Conversely, mutation of Thr136 (T136), Arg140 (R140A), or Phe142 (F142A) maintained the agonist-induced increase in hIP:Rab11a interaction (Figure 2B, upper panels & Supplemental Figure 3, upper panels). In all cases, differences in the levels of the Rab11a, or of its Ala-scanning variant proteins, in the anti-HA immunoprecipitates were not due to variations in the efficiency of the immunoprecipitation of the hIP per se (Figure 2B, middle panels & Supplemental Figure 3, middle panels), or in their respective expression levels (Figure 2B, lower panels & Supplemental Figure 3, lower panels). In addition, none of the Rab11a variant proteins were detected in the corresponding anti-HA precipitates from the control HEK.β-Gal cell line, thereby further confirming specificity (Figure 2B, upper panels).

3.3 Assessment of the hIP:Rab11a interaction through Surface Plasmon Resonance
To further investigate the association of Rab11a with the hIP, surface plasmon resonance (SPR) was also performed to accurately measure the in vitro biophysical binding properties of the wild-type Rab11a, and each of the Ala-scanning α4 helix variants, to a peptide corresponding to the RBD spanning residues 296-312 of the hIP (pepIP296-312, Figure 3). Initially, a binding screen was carried out whereby the chip-immobilised hIP peptide was screened with single concentrations (2 μM) of the wild-type and Ala-scanning variants of Rab11a (Figure 3A). The majority of the Rab11a variants bound the hIP peptide at levels less than that of the wild-type Rab11a, while the Phe142 (F142A) variant actually bound at higher levels (Figure 3A & Table 1A). Consistent with the
aforementioned findings from the Ala-scanning studies in Y2H and co-immunoprecipitations in mammalian cells (Figures 2A & 2B), it was notable that mutation of the acidic residues, in particular Glu\textsuperscript{138} (E138A) and Glu\textsuperscript{144} (E144A), resulted in the greatest reduction in the associations of the hIP peptide (Figure 3A). Hence, a detailed kinetic characterisation of the interaction of the hIP peptide (pepIP\textsuperscript{296-312}) with the wild-type Rab11a and the Glu\textsuperscript{138} (E138A) and Glu\textsuperscript{144} (E144A) variants using three different concentrations (1, 2 & 4 μM) of the Rab11a protein was subsequently carried out (Figure 3B & Table 1B). Binding of the wild-type Rab11a to the hIP peptide was highly reproducible, with an apparent K\textsubscript{D} of ~241 nM (Figure 3B & Table 1B). Consistent with the binding screens (Figure 3A), the Glu\textsuperscript{138} (E138A) and Glu\textsuperscript{144} (E144A) variants of Rab11a displayed significantly decreased binding affinities with an apparent K\textsubscript{D} determined to be ~1.03 μM and ~324 nM, respectively (Figure 3B & Table 1B). In addition, to investigate the possible additive effect of dual Glu\textsuperscript{138} and Glu\textsuperscript{144} mutation, binding screens of the hIP peptide (pepIP\textsuperscript{296-312}) with single concentrations (1 μM) of the wild-type Rab11a, the individual Glu\textsuperscript{138} (E138A; Rab11a\textsuperscript{E138A}), individual Glu\textsuperscript{144} (E144A; Rab11a\textsuperscript{E144A}), or the dual Glu\textsuperscript{138} & Glu\textsuperscript{144} (E138,144A; Rab11a\textsuperscript{E138A,E144A}) Ala-scanning variant proteins were also performed (Supplemental Figure 4). Consistent with previous screens (Figure 3A & 3B), while the Glu\textsuperscript{138} (E138A) and Glu\textsuperscript{144} (E144A) variants displayed decreased binding levels relative to the wild-type Rab11a, binding of the dual Glu\textsuperscript{138} & Glu\textsuperscript{144} (E138,144A) variant was similar to that of the Glu\textsuperscript{138} (E138A) variant alone with no additive impact on the interaction with the pepIP\textsuperscript{296-312} peptide apparent (Supplemental Figure 4). In all cases, the purity of Rab11a and its Ala-scanning variants were confirmed by SDS-PAGE while far-UV circular dichroism (CD) was used to validate Rab11a protein folding (Supplemental Figure 5A & 5B). Consistent with previously published data on Rab11a [44], the CD spectral analyses confirmed the presence of α-helical and β-sheet secondary structure with no significant deviations between the wild-type Rab11a and the Ala-scanning variants observed (Supplemental Figure 5B).

Taken together, these data demonstrate a novel role for the α4 helix of Rab11a in mediating its association with the hIP. Complementary Ala-scanning mutagenesis approaches, within both yeast and mammalian cells, along with SPR biophysical characterisations indicate that certain charged residues within the α4 helix of Rab11a, namely Glu\textsuperscript{138}, Glu\textsuperscript{144} and to a lesser extent Lys\textsuperscript{145}, may be key determinants in mediating the interaction of Rab11a with the hIP. In particular, these residues also appear to play a fundamental role in the agonist-induced increase in interaction of Rab11a with the hIP.

### 3.4 Homology modelling of the inactive and active states of the hIP

Recent advances in elucidating the structures of GPCRs have shed new light into understanding the mechanisms of receptor activation [45, 46]. The two-state equilibrium model of GPCR activation proposes that the receptor essentially exists in one of two distinct conformations, the inactive state or the active state [45, 46]. In brief, superimposing the models for both the ‘inactive’ and ‘active’ states reveal only moderate structural differences within their extracellular domains [38, 39]. In contrast, the cytoplasmic regions show a number of agonist-induced structural rearrangements between the inactive and active states, with the most significant shift being the outward movement or displacement of the cytoplasmic end of transmembrane domain (TM) 6 in response to agonist-activation [38, 39]. Moreover, this displacement of TM6 in the active-state model is coupled with the outward movement of TM5, as well as the inward movement of TM7. The movement of TM7 is due to the accommodation of the bulky hydrophobic side-chain of the Tyr residue within the NPXXY sequence found on many, but not all, GPCRs. Such activation-status dependent conformational changes in the cytoplasmic interface of TM5, TM6 and TM7 are also proposed to affect the structural orientation(s) and/or interactions with the α-H8 domain located at the proximal end of the C-tail of most class A/rhodopsin-like GPCRs [38, 39, 47]. Notably, in the case of the hIP, as the α-H8 and adjacent palmitoylated cysteines, namely Cys\textsuperscript{308}, Cys\textsuperscript{309} and
Cys^{311}, play a major functional role in the hIP by acting as an agonist-regulated Rab11a-binding domain (RBD) [6], it is entirely theoretically possible that such agonist-induced conformational changes within the α-H8 may indeed affect, or even explain, the binding of Rab11a with the hIP.

Thus, while crystal structures for the hIP have not yet been resolved, the recent availability of structures for several rhodopsin-like/class A GPCRs, including in their inactive and activated states, makes it possible to generate good homology models of the hIP and then thereafter, to attempt in silico macromolecular docking of the hIP in complex with Rab11a. Hence, herein, the I-TASSER algorithm [34, 35] was used to generate homology models of the hIP simulated in both its ‘inactive’ and ‘active’ states. Thus, to this end, to generate the model for the inactive hIP (Figure 4A, left panel), the hIP sequence was threaded onto the resolved carazolol-bound β2AR structure (PDB: 2RH1, [36]). Similarly, to generate the proposed active state model of the hIP (Figure 4A, right panel), the template used was the agonist (B1167107)-bound β2AR (PDB: 3POG, [38]).

Consistent with the data reported for the β2AR, A_{2a}R and rhodopsin/opsin [38, 39, 47], superimposition of the simulated models of the hIP suggests that only moderate structural differences occur between the inactive and active state models within the extracellular domain (Figure 4B), while the major agonist-induced structural rearrangements occur within the cytoplasmic region (Figure 4C). Of note, in the prostanoid receptor subfamily of GPCRs, the NPXXY sequence at the cytoplasmic end of TM7 is replaced by the variant DPXXF sequence, or specifically in the case of the hIP, D^{298}PXXF^{302}. Within the active state homology model of the hIP, it is predicted that the significant outward shifts of the cytoplasmic ends of TM5 and TM6 are coupled with an inward movement of TM7, due to the accommodation of the bulky hydrophobic side-chain of Phe^{292} (Figure 4D). The flexibility of TM7, due to movement of the DPXXF motif, is coupled with a movement and rotation of α-H8. This predicted agonist-induced movement of the α-H8 region is particularly noteworthy in the case of the hIP as the α-H8 region, as previously stated, also corresponds to the RBD that mediates interaction of the hIP with Rab11a (Figure 4D). Based on these in silico homology model predictions, combined with previous detailed molecular characterisations of hIP RBD [6], it is proposed that this rearrangement of the RBD, largely composed of the α-H8 domain corresponding to Val^{299}-Val^{307}, together with deacylation of the adjacent Cys residues (Cys^{308}, Cys^{309} and Cys^{311}), may represent a structural basis to explain the agonist-induced increase in the interaction of the RBD of the hIP with Rab11a.

### 3.5 Macromolecular modelling of the putative hIP:Rab11a complex

Hence, employing the simulated homology models for the inactive and agonist-bound activation states of the hIP, along with input from specific experimental data identifying key residues within the hIP:Rab11a binding interfaces, in silico macromolecular docking was used to model the interaction of the hIP in complex with Rab11a. Molecular docking was performed using the HADDOCK (High Ambiguity Driven DOCKing) package [41], as it can simulate backbone flexibility and incorporate side-chain optimisation into the docking procedure, unlike many other docking algorithms currently available [48–50]. In addition, in generating the macromolecular complexes, HADDOCK allows the user specify key residues thought to be important for the interaction such as those residues identified as important through experimental approaches, as outlined herein.

For docking, only residues 1-336 of the hIP were included in the modelled structures submitted to HADDOCK as these were the regions that had the highest degree of structural confidence, as determined by Ramachandran plot analysis (data not shown). Similarly, the homology model for Rab11a was truncated at residue 188, within its HVD. To guide the docking algorithm, residues previously determined to be important for the interaction, namely Phe^{309}, Arg^{302}, Leu^{303}, Lys^{304}, Leu^{305} and Val^{307} within the α-H8/RBD of the hIP [6], along with Asp^{137}, Glu^{138}, Glu^{144} and Lys^{145} within the α4 helix of Rab11a (Figure 2 & 3, Table 1; this study),
were specified as being potentially active residues involved in hIP:Rab11a binding. As previously stated, Cys$^{308}$ and Cys$^{309}$ of the hIP have been determined to play a critical role in the agonist-induced interaction with Rab11a [6]. More specifically, agonist-regulated deacetylation of Cys$^{308}$ and Cys$^{309}$ is thought to orientate and position the essential residues of the α-H8/RBD in proximity to Rab11a to facilitate interaction of the hIP RBD with Rab11a [6]. Therefore, for docking of the active-state homology model of the hIP with Rab11a, Cys$^{308}$ and Cys$^{309}$ were also specified as potentially active residues that may contribute to, or influence, the interaction between the hIP and Rab11a. Using these specified parameters, the highest-ranked structures generated for the inactive hIP:Rab11a and active hIP:Rab11a complexes are shown in Figure 5A and Figure 5B, respectively. In comparing these two highest-ranked simulations, it is indeed notable that, owing to structural differences within the hIP RBD between the inactive- versus agonist-activated hIP [6], there are radical differences in the simulated interactions between the hIP with Rab11a depending on the activation-status of the receptor (Figure 5A and Figure 5B). Most critically, in the active-state model, Rab11a is predicted to be rotated approximately 50° about the hIP relative to the position of Rab11a in the inactive-state model (Figure 5A and Figure 5B).

Hence, PyMOL was next used to find and display all polar contacts in both the inactive (Figure 5C) and agonist-activated (Figure 5D) forms of the hIP that lie within the acceptable range for hydrogen bonding of 2.5-3.5 Å [51] enabling complex formation between the RBD of the hIP with α4 helix of Rab11a. Employing these parameters, within the inactive hIP:Rab11a complex, Lys$^{304}$ within the RBD of the hIP is predicted to make a contact with Glu$^{138}$ within the α4 helix of Rab11a (Figure 5C). The fact that experimental data shows that Rab11a$^{E138A}$ can associate with the hIP in the absence of agonist suggests that, in addition to the Glu$^{138}$:Lys$^{304}$, other contacts between the Rab11a:hIP complex plays a key role in their constitutive interaction (Figure 2B & 2C). Furthermore, within the modelled active hIP:Rab11a complex, while this same Lys$^{304}$:Glu$^{138}$ contact is also strongly implicated, there are also proposed contacts made between Cys$^{309}$ within the RBD of the hIP and Glu$^{138}$ within the α4 helix of Rab11a, in addition to a contact between the main chain carbonyl oxygen of Leu$^{310}$ of the hIP with Lys$^{145}$ of Rab11a (Figure 5D). These proposed additional contacts within the active hIP:Rab11a are due to a differential positioning of Rab11a relative to the RBD of the hIP within the inactive- versus agonist-activated hIP and are likely to account for the enhanced interaction between the hIP with Rab11a that occurs in response to agonist-activation (Figure 2B & 2C). Thus, and as presented in Figure 5E, such marked differences between the two modes of binding of Rab11a to the hIP are most optimally illustrated by superimposing the inactive- and active- homology models of the hIP. Most strikingly, within the active hIP:Rab11a complex (pink:orange, respectively; Figure 5E), Rab11a is rotated approximately 50° about a vertical axis through the hIP, relative to the position of Rab11a within the inactive hIP:Rab11a complex (green:blue, respectively; Figure 5E). Such a rotation in Rab11a accounts for the increased contacts between Glu$^{138}$:Lys$^{304}$, Glu$^{138}$:Cys$^{309}$ and Lys$^{145}$:Leu$^{310}$ observed with the active hIP, in addition to the contacts found with the inactive hIP:Rab11a complex, and thereby explains the constitutive interaction of Rab11a with the hIP in the absence of agonist that is further enhanced in the active hIP:Rab11a complex in response to agonist-activation (Figure 2B & 2C).

3.6 Role of Glu$^{138}$:Lys$^{304}$ in mediating the constitutive and agonist-induced Rab11a:hIP interactions.

In order to further test this proposed mechanism experimentally, the importance of the contacts between Glu$^{138}$:Lys$^{304}$ within the Rab11a:hIP inactive/constitutive and agonist-activated complexes was examined through additional co-immunoprecipitation studies between wild type Rab11a and its variant Rab11a$^{E138A}$ with either the hIP, hIP$^{K304A}$ or hIP$^{K304Q}$. In the latter mutations, the critical Lys$^{304}$ within the RBD of the hIP was mutated to either an Ala$^{304}$ or by a semi-conservative substitution to a Gln$^{304}$, thereby minimizing any potential non-specific mutational effects due to residue size. Consistent with previous findings, in the absence of agonist,
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

the hIP showed a strong constitutive association with Rab11a that was further enhanced following agonist (cicaprost)-activation of the receptor (Figure 6A & 6B). However, mutation of Lys$^{304}$ of the hIP to either an Ala (hIP$^{K304A}$) or to a Gln (hIP$^{K304Q}$) each substantially reduced the constitutive interaction with Rab11a and each also abolished the agonist-induced increase in association between the hIP with Rab11a (Figure 6A & 6B). Moreover, the critical and specific requirement for a Lys at position 304 within the hIP for the interaction with Rab11a was further established whereby mutation of Lys$^{304}$ to either an Ala (hIP$^{K304A}$) or to a Gln (hIP$^{K304Q}$) each substantially reduced the constitutive interaction with Rab11a and each also abolished the agonist-induced association between the hIP with Rab11a (Figure 6A & 6B). Hence, substituting Lys$^{304}$ with an Arg$^{304}$ cannot compensate for the loss of constitutive or agonist-induced interactions between the hIP with Rab11a.

Moreover and consistent with previous data (Figure 2B & 2C), while mutation of Glu$^{138}$ within Rab11a (E138A) did not appear to affect the constitutive interaction between Rab11a$^{E138A}$ with the hIP in the absence of agonist, this mutation almost completely abolished the interaction, including agonist-enhanced interaction, with the hIP in the presence of agonist (Figure 6C & 6D). As previously stated, the fact that Rab11a$^{E138A}$ can associate with the hIP in the absence of agonist suggests that in addition to the Glu$^{138}$:Lys$^{304}$ other contacts between the Rab11a:hIP complex plays a key role in their constitutive interaction. Notably, the E138A mutation within Rab11a$^{E138A}$ virtually abolished both the constitutive and agonist-induced interaction with the variant forms of the hIP whereby the critical Lys$^{304}$ within its RBD was mutated and irrespective of whether it was converted to either to an Ala (hIP$^{K304A}$), Gln (hIP$^{K304Q}$) or to a positively charged Arg (hIP$^{K304R}$) residue.

Collectively, data presented herein identify a novel binding site corresponding to the α4 helix of Rab11a involved in its interaction within the hIP, a site that has not been previously identified as a protein binding site within Rab11a and a site that is entirely distinct from the Switch I and Switch II regions identified as the specific interacting domains for most other Rab/Ras-like interactants to date [31, 32]. In addition, homology modelling and molecular docking simulations suggest two distinct structural binding modes for Rab11a with the hIP that are dependent on the activation status of the receptor. As stated, it has been previously proposed that agonist-activation of the hIP, in combination with agonist-regulated deacylation at Cys$^{308}$/Cys$^{309}$, results in a conformational rearrangement to orientate the critical residues within the α-H8/RBD of the activated hIP in proximity to Rab11a to facilitate interaction of the hIP with Rab11a [6]. Consistent with this proposal, data generated herein from molecular docking and mutational studies suggests that this rearrangement, in turn, leads to an altered binding interface for Rab11a with the hIP. While the altered interaction of Rab11a with the agonist-activated hIP is still proposed to be mediated by residues within the α4 helix of Rab11a and the RBD of the hIP, modelling simulations suggest that there is a profound difference in the positioning and presentation of the Rab11a protein within the active state hIP:Rab11a complex. Hence, the experimental and in silico studies presented herein provide a molecular basis to explain the constitutive and agonist-dependent interaction between the hIP and Rab11a, primarily involving an interaction between their RBD and α4 helix domains, respectively.
4 DISCUSSION

In the present study, the regions of Rab11a required for its interaction with the hIP were investigated. Through deletional approaches and Y2H-based interaction studies, the α4 helix region of Rab11a, spanning residues 136-145, was identified as a critical region involved in the interaction with the RBD of the hIP. Deletion of this region within Rab11a, originally identified from the initial Y2H library screen, demonstrated a specific requirement for the α4 helix region, at least, to maintain an interaction with all bait proteins containing the RBD of the hIP. Moreover, and notably, the hIP subfragment containing only the RBD (α-H8 and adjacent Cys308-311 residues) but devoid of the remaining C-tail domain of the hIP was sufficient to support the interaction with the Rab11a prey protein. In addition, through complementary mutational approaches in both Y2H-based studies and co-immunoprecipitations of the full-length hIP and Rab11a proteins in mammalian cells, supported by in vitro biophysical characterisations employing SPR, a number of charged residues within the α4 helix of Rab11a, namely Asp137, Glu138, Glu144 and Lys145, were identified as necessary for the agonist-induced interaction with the hIP. This agonist-induced increase in the interaction between hIP and Rab11a has been previously shown to be maximal at 2 hr post-cicaprost treatment, and coincides with the time of maximal agonist-induced internalization of the hIP [6, 11]. While these investigations cannot completely exclude the possibility that other regions within Rab11a may also potentially contribute to the interaction with the hIP, the finding that even on mutation of single residues within the full length Rab11a, e.g E138A or E144A, resulted in loss of the agonist-induced Rab11a:hIP complex in co-immunoprecipitations in mammalian cells suggests that the major, or even sole, domain of contact between the receptor and the GTPase is via the α4 helix. Further credence to our finding that the hIP RBD is not binding to the SwitchI/SwitchII domains but rather to the α4 helix is provided by fact that it has been previously shown that receptor binding to Rab11a occurs independently of its guanine nucleotide (GDP/GTP)-status [11]. Hence, the findings herein represent the identification of a novel binding region within Rab11a, namely its α4 helix, a protein binding site that is entirely distinct from the binding regions identified for most other Ras-like GTPase interactants [31, 32].

Understanding the particular dynamics of a protein-protein interaction is critical for a greater appreciation of the complex cellular signalling or regulatory pathways mediated by that interaction. The recently reported structure of the agonist-bound β2AR in complex with G, has revealed in elaborate detail the molecular interplay between a GPCR and its defining interactants, namely the Ga and Gβγ constituents of the heterotrimeric G protein complex [52]. This structure sheds significant insight into the receptor’s workings, specifically on how agonist-induced conformational changes are transduced through rearrangement of the cytoplasmic domains, leading to significant movements within the bound Gaα subunit. This mechanism is proposed to facilitate guanine nucleotide-exchange and subsequent activation of the G protein and its associated signalling pathways. Herein, in silico computational modelling of the hIP:Rab11a complex was used to simulate the putative interface mediated by residues within the RBD of the hIP and the α4 helix of Rab11a. Notably, this in silico approach suggested differing binding modes for Rab11a that are mediated by agonist-activation of the hIP. Within the inactive hIP:Rab11a complex, such macromolecular modelling and docking predicts that contacts between Lys304 of the hIP with Glu138 of Rab11a may be critical in stabilising the constitutive interaction. Agonist-induced conformational changes within the receptor structure, in particular in the TM7/α-H8 region, in addition to agonist-induced deacylation at Cys308/Cys309, and the subsequent availability of these residues to contribute to the interaction, suggest a further binding mode for Rab11a within the active hIP:Rab11a complex, involving contacts between Lys304, Cys309 and Leu110 of the hIP with Glu138 and Lys145 of the α4 helix of Rab11a. It is essential to note that these hIP:Rab11a complexes generated based on in silico homology modelling and docking analyses, albeit while incorporating a significant amount of experimental data into their
generation and validation, may not necessarily identify all molecular contacts between the two interacting proteins. In all molecular docking simulations, the influence of the lipid membrane is not accounted for, and the homology models used in these simulations did not include explicit solvent. Hence, any failure of the simulated models to demonstrate direct roles in contact formation for certain residues within the RBD of the hIP or the α4 helix of Rab11a (e.g. Glu\[138\], Lys\[145\] or Asp\[135\]) experimentally established to be required for the interaction may simply be due to those contacts being mediated through solvent molecules within the physiological complex. In addition, the template structures for the GPCRs used in the generation of the homology models of the hIP herein represent only a single possible conformation of the respective receptors. GPCRs are remarkably dynamic proteins, and the structures solved for these receptors correspond to a particular conformational state whose stability is amenable to crystallisation. Bearing in mind the fluid nature of GPCR activation, other less-stable conformations have also been proposed to be important for function [53].

Validation of the importance of the predicted contacts between Glu\[138\]:Lys\[304\] within the Rab11a:hIP inactive/constitutive and agonist-activated complexes was established experimentally through additional co-immunoprecipitation studies between wild type Rab11a and its variant Rab11a\[E138A\] with either the hIP, hIP\[K304A\], hIP\[K304Q\] or hIP\[K304R\]. While mutation of Glu\[138\] within Rab11a (E138A) did not significantly affect the constitutive interaction between Rab11a\[E138A\] with the hIP, this mutation almost completely abolished the interaction, including agonist-enhanced interaction, with the hIP in the presence of agonist. These findings suggest that while Glu\[138\] within Rab11a may not be the sole determinant for interaction of Rab11a with the inactive hIP, it is critically required to stabilise the enhanced interaction of Rab11a with the active hIP. Moreover, the E138A mutation within Rab11a\[E138A\] virtually abolished both the constitutive and agonist-induced interaction with the variant forms of the hIP whereby the critical Lys\[304\] within its RBD was mutated and irrespective of whether it was converted to either an Ala (hIP\[K304A\]) or Gln (hIP\[K304Q\]). The critical and specific requirement for a Lys at position 304 within the hIP for the interaction with Rab11a was further established whereby mutation of Lys\[304\] to a positively charged Arg\[304\] within the hIP\[K304R\] variant resulted in a significantly reduced constitutive interaction with Rab11a while the enhanced agonist-induced association between the hIP\[K304R\] with Rab11a was abolished. It is indeed notable that hIP has been previously established to contain a novel evolutionary conserved ER export motif, defined by “K/R(X)\[297\]K/R(X)K/R\[302\]”, where Lys\[297\], Arg\[302\] and Lys\[304\] within its α-H8 domain form the essential core residues of that ER export motif [7]. Critically, while substituting Lys\[304\] with a positively charged Arg\[304\] cannot compensate for the loss of constitutive or agonist-induced interactions between the RBD of the hIP with Rab11a, as determined in the current study (Figure 6), this semi-conservative Lys\[304\] to Arg\[304\] substitution does serve as a functional substitution within the ER export motif of the hIP [7]. Thus, while certain residues within the α-H8 domain of the hIP can serve as part of an ER export motif involved in the processing and maturation of the newly synthesized receptor as it exits the ER [7], key residues within this same α-H8 domain along with adjacent palmitoylable Cys residues (Cys\[308\], Cys\[309\] and Cys\[311\]) also form an essential component of the RBD involved in the intracellular trafficking of the mature hIP in response to agonist activation.

Despite several attempts, co-crystallisation of the Rab11a protein and a hIP peptide based on the RBD of the hIP (pephIP\[296-312\]), and the subsequent determination of the structure of that hIP:Rab11a complex was not successful where any crystals generated were deemed too small for diffraction (Khan, Mulvaney & Kinsella, data not shown). While there is no doubt that such crystallographic data has the potential to shed significant additional molecular insight into the interaction between Rab11a and the hIP RBD, even in the absence of such detailed structural information a number of hypotheses can none-the-less be proposed. One intriguing possibility is that the positioning and presentation of the Rab11a protein within the active hIP:Rab11a complex may facilitate the nucleotide-exchange and activation of Rab11a, through the recruitment and engagement of
specific RabGEFs. A similar mechanism has been proposed for the AT1aR-Rab5a complex, wherein agonist stimulation of the receptor directly promoted the nucleotide-exchange and activation of Rab5a [54]. A further possibility is that the change in positioning and presentation of Rab11a within the active hIP-Rab11a complex may facilitate the recruitment of specific Rab effectors involved in the progression of the hIP through the endocytic pathway. Such possibilities are consistent with the increasing body of evidence that indicates that GPCRs may not simply be “passive cargo molecules”. Rather, GPCR activation has been shown to directly influence the activity of Rab proteins and their associated regulatory proteins, and it is proposed that GPCRs may contribute significantly to the regulation of their own trafficking within the endocytic pathway [55-59].

Numerous studies have focused on the structural characterisation of effectors and other regulatory proteins that complex with members of the Rab GTPase family. Most notably, these include Rab GTPase-activating protein (RabGAP), Rab guanine nucleotide dissociation inhibitor (RabGDI), Rab guanine nucleotide exchange factor (RabGEF), Rab escort protein (REP), and members of the FIPs [32, 60-63]. Analysis of the binding interfaces within these complexes has significantly improved the understanding of Rab regulatory mechanisms, and how Rab proteins can achieve specificity in membrane trafficking. Within each of the complexes that have been characterised to date, the interaction between the Rab protein and its partner is predominantly mediated through the Switch I and Switch II domains (Figure 7A). In addition, in the case of RabGDI and REP, while there are also significant contacts with the HVD (Figure 7A), these contacts are localised to the isoprenylated C-terminal membrane association domain (MAD) of the Rab proteins. These additional contacts reflect the role for RabGDI as a solubilising/recycling factor, and for REP as an integral part of the Rab geranylgeranyl transferase II and its catalysis of Rab isoprenylation [64, 65]. Hence, as depicted in the models presented in Figure 7B & 7C, the discoveries herein represent the very first identification of α4 helix as a novel binding domain within Rab11a, that is uniquely distinct from these latter GTPase effector-binding domains, to mediate the interaction with the RBD located within the C-tail of the hIP. Notably, the fact that Rab11åE138A variant does associate with the wild type hIP in the inactive/resting state, in the absence of agonist, but does not associate with either the agonist-activated wild type hIP or with its hIPK304A, hIPK304Q or hIPK364A variants either in the absence or presence of agonist (Figure 6) suggests that Glu138 on Rab11a is not the sole determining residue required for interaction with the hIP, as depicted in the models (Figure 7B & 7C).

With regard to RBDs within other GPCRs that have been characterised to date, they also share a similar requirement for basic (Lys/Arg) residues within the RBD itself, and/or a positioning adjacent to palmitoylatable cysteines [12-14]. Hence, though as yet to be investigated, it is possible that the α4 helix of Rab11a may also act as a common binding site for the RBDs of these receptors, similar to that determined herein for the hIP. As such, the RBD:α4 helix interface may serve as a novel drug target for development of specific inhibitors to disrupt the interaction of a particular receptor with Rab11a in clinically significant contexts. Moreover, while members of the Rab11 subfamily of GTPases, consisting of the ubiquitously expressed Rab11a, Rab11b and the epithelial Rab25, act as principal regulators of membrane trafficking from the endosomal-recycling compartment [66, 67], they themselves are increasingly implicated in angiogenesis [68] and, along with Rab5a and Rab21, in cancer cell migration and invasiveness through regulation of α-integrin signalling [69]. Hence, the discoveries herein of a novel binding platform on the Rab GTPases, in this case the α4 helix of Rab11a, sheds significant new light into their functional capacity for protein binding. In terms of the repertoire of binding partners, whether binding of α4 helix is confined to the hIP or whether it might extend to other GPCRs or indeed to other types/classes of interacting partners clearly needs to be investigated a priori.

In conclusion, this study provides evidence of a novel binding site for the hIP within Rab11a. Findings from these studies point to a critical role played by the α4 helix of Rab11a in mediating the interaction of Rab11a with the hIP. Furthermore, in silico computational modelling of the hIP-Rab11a complex supported by
substantial experimental evidence suggests variable binding modes for Rab11a within the complex, which are dependent upon the activation status the receptor (inactive versus agonist-activated). These studies provide further mechanistic insight into the intracellular trafficking and recycling of the hIP and, potentially, of other members of the GPCR superfamily. Bearing in mind that GPCR activation has been implicated in influencing the activity of Rab proteins with which they interact, these data shed new insights into the mechanisms that GPCRs may employ to directly regulate their own intracellular trafficking.

ACKNOWLEDGEMENTS
This work was supported by Science Foundation of Ireland (Grant SFI: 05/IN.1/B19) awarded to B.T.K.

ABBREVIATIONS
GPCR, G protein-coupled receptor; IP, I prostanoid receptor/prostacyclin receptor; hIP, human IP; α-H8, α-helix 8; α4, Rab α-helix 4; C-tail, carboxyl-terminal tail; FIPs, family of Rab11 interacting proteins; GFP, green fluorescent protein; HA, hemagglutinin; HEK, human embryonic kidney; RBD, Rab11 binding domain; TM, transmembrane; Y2H, yeast two-hybrid; DDO, double drop-out; QDO, quadruple drop-out; MEM, minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; WT, wild type; PGI2, prostaglandin I2/prostacyclin; HVD, hypervariable domain; SDM, site-directed mutagenesis; CD, circular dichroism; SPR, surface plasmon resonance; PDB, Protein Data Bank; I-TASSER, Iterative-Threading ASSEmblY Refinement, MAD, membrane association domain; HADDOCK, High Ambiguity Driven DOCKing; RabGAP, Rab GTPase-activating protein; RabGDI, Rab guanine nucleotide dissociation inhibitor; RabGEF, Rab guanine nucleotide exchange factor; REP, Rab escort protein.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
All authors contributed materially to the work presented in this paper. E.P.M., F.M., A.R.K., D.J.O’C. and B.T.K. designed the experiments. E.P.M. performed the Y2H and co-immunoprecipitation experiments and the in silico modelling and docking analysis. E.P.M., F.M. and D.J.O’C. performed the protein purification and SPR experimentation and analysed the data. A.R.K. & E.P.M performed the crystallization screens. E.P.M. and B.T.K. wrote the paper. All the authors have read and approved the final manuscript.
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[56] N. Charette, P. Holland, J. Frazer, H. Allen, D.J. Dupre, Dependence on different Rab GTPases for the trafficking of CXCR4 and CCR5 homo or heterodimers between the endoplasmic reticulum and plasma membrane in Jurkat cells, Cell Signal, 23 (2011) 1738-1749.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

Table 1: Surface plasmon resonance Assessment of Rab11a binding to the RBD of the hIP (pepIP^{286-312}).

Table 1A: Binding Screen.

<table>
<thead>
<tr>
<th>Rab11a Variant</th>
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<th>Dissociation Rate, kd* (1/s)</th>
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<td>Rab11a^{E142A}</td>
<td>363</td>
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<td>Rab11a (WT)</td>
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</tr>
<tr>
<td>Rab11a^{R140A}</td>
<td>302</td>
<td>8.34E-05</td>
</tr>
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<tr>
<td>Rab11a^{E144A}</td>
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*Maximum quantities bound (Response Units, RU) and Dissociation Rates (kd) from SPR binding screens (using 2 μM Rab11a protein, as listed) were calculated using GE Healthcare Biaevaluate analysis software.

Table 1B: Kinetic Screen.

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$ Binding affinity rate constants (Kd) from kinetic characterisations (using 1, 2 & 4 μM Rab11a protein, as listed) were calculated using GE Healthcare Biaevaluate analysis software, where data were fitted to a 1:1 Langmuir model.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

FIGURES & FIGURE LEGENDS

**Figure 1: Interaction of Rab11a with the hIP**

*Panel A:* Homology model of the full-length Rab11a generated using the I-TASSER algorithm [34, 35]. A linear schematic is also presented for full-length Rab11a and the Rab11a<sub>124-216</sub> prey protein identified to interact with the hIP through Y2H screening. In the schematic, the labels represent the approximate boundaries of the secondary structure elements. The colouring scheme of the elements used in the linear representation corresponds to that used in the three-dimensional structure. *Panel B:* S. cerevisiae Y187 (pACT2:Rab11a<sub>124-216</sub>), or S. cerevisiae Y187 strains harbouring the listed N (amino)-terminal truncated deletions of Rab11a<sub>124-216</sub>, were mated with S. cerevisiae AH109 bait strains transformed with recombinant pGBKT7 encoding the listed subfragments of the hIP and, as controls, S. cerevisiae AH109 (pGBKT7:p53) or S. cerevisiae AH109 (pGBKT7) (Ø). Diploids were selected by growth on DDO medium, whereas interactants were selected on QDO medium, and by their ability to express β-Gal. Data presented are representative of at least three independent experiments (n ≥ 3). Models of the structure of Rab11a<sub>124-216</sub> showing the boundaries of secondary structure used to generate N-terminal truncated Y2H fragments are shown alongside each panel.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

Figure 2: Effect of Ala-scanning mutagenesis of the α4 helix of Rab11a on its interaction with the hIP

Panel A: S. cerevisiae Y187 (pACT2:Rab11a124-216) (WT) or S. cerevisiae Y187 prey strains encoding the listed Ala-scanning variants of the α4 helix region of Rab11a, were mated with S. cerevisiae AH109 bait strains transformed with recombinant pGBK7 encoding the listed subfragments of the hIP and, as controls, S. cerevisiae AH109 (pGBK7:p53) or S. cerevisiae AH109 (pGBK7) (Ø). Diploids were selected by growth on DDO medium, whereas interactants were selected on QDO medium, and by their ability to express β-Gal, as a result of GAL4-dependent transcriptional activation of the HIS3, ADE2 and lacZ reporter genes, respectively, due to interaction between bait and prey proteins. Data presented are representative of at least three independent experiments (n ≥ 3). All Rab11a prey proteins listed (Rab11aWT and Rab11aT137A-Rab11aK145A) correspond to Rab11a124-216 subdomain as opposed to the full length Rab11a1-216.

Panel B: HEK.hIPWT or, as control, HEK.β-Gal cells, each transiently transfected with pEGFP-C1 encoding GFP-tagged Rab11a, or the listed Ala-scanning variants of the α4 helix region of Rab11a, were incubated with vehicle (0) or 1 μM cicaprost for 2 hr, prior to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE, and subject to successive immunoblotting (IB) versus anti-GFP antibody (upper panels) or anti-HA 3F10 HRP-conjugated antibody (middle panels). To verify expression of the Rab proteins, aliquots of whole cell lysate (50 μg/lane) were immunoblotted versus anti-GFP antibody (lower panels). The relative positions of the molecular size markers (kDa) are indicated to the left of the panels. Panel C: The bar chart shows the mean agonist-induced fold increase in the levels of the respective Rab proteins associated with the immunoprecipitates of the hIP, as determined by quantitative densitometry (± SEM, n ≥ 3). The asterisks indicate where mutation resulted in a significant change in the agonist-induced fold increase in interaction, compared to the wild-type Rab11a, where *, **, and *** indicate p < 0.05, p < 0.01 and p < 0.001, respectively (Dunnett's multiple comparison post hoc test following one-way ANOVA).
Figure 3: Biophysical characterisation of the interaction of Rab11a with the hIP

Representative SPR sensorgrams from: Panel A, Binding screens of the purified wild-type Rab11a (WT) and its listed Ala-scanning variant proteins were screened vs the immobilized hIP RBD peptide (residues 296-312; pepIP296-312). Rab11a proteins were injected at 2 μM for 30 min over a Series S streptavidin-coated (SA) sensor chip with immobilized pepIP296-312 followed by buffer flow for 90 min. Panel B, Kinetic characterisations of the purified Rab11a (WT), and the listed Ala-scanning variant proteins screened versus with the immobilized hIP peptide (pepIP296-312). Rab11a proteins were injected at 1, 2 & 4 μM for 30 min over a Series S streptavidin-coated (SA) sensor chip with immobilized pepIP296-312 followed by buffer flow for 90 min. Maximum quantities bound (Response Units, RU) and Dissociation Rates (kd) from the binding screens in Panel A, calculated using GE Healthcare Biaevaluate analysis software, are given in Table 1A. Binding affinity rate constants (KD) from kinetic characterisations in Panel B, calculated using GE Healthcare Biaevaluate analysis software where data were fitted to a 1:1 Langmuir model, are given in Table 1B.
Figure 4: Homology modelling of the inactive and active state structures of the hIP

Panel A: Homology models for the proposed inactive state (green; left panel) and agonist-bound, active state (pink; right panel) structures of the hIP were generated using the I-TASSER algorithm [34, 35]. Panels B - D: Structural alignment of the homology models for the proposed inactive and active state structures of the hIP.

Panel B: A view from the extracellular side/face of the aligned models, showing minimal structural changes between the inactive and active states. Panel C: A view from the intracellular (cytoplasmic) side/face of the aligned models, showing the significant agonist-induced structural changes between the inactive and active states. These changes include the outward movement or displacement of TM6 (dashed red arrow), coupled with the outward movement of TM5 and inward movement of TM7. Panel D: The significant outward movement of TM6 is coupled with an inward movement of TM7 (green arrow), due to the accommodation of the bulky hydrophobic side-chain of Phe292 of the DPXXF sequence. As a consequence of the rearrangement of TM7, there is also a significant movement/rotation of α-H8, containing the RBD of the hIP.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

**Figure 5: Macromolecular modelling of the hIP:Rab11a complex**

Panel A: HADDOCK docking complex for the inactive hIP (residues 1-336; green) and Rab11a (residues 1-188; blue) homology model structures. Panel B: HADDOCK docking complex for the active hIP (residues 1-336; pink) and Rab11a (residues 1-188; orange) homology model structures. Panel C: Cartoon model depicting the interaction between the RBD of the hIP (residues 299-312) with the α4 helix domain of Rab11a (residues
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

136-145), where the structure of the RBD is equivalent/derived from that proposed for the inactive state model of the hIP. PyMOL was used to identify potential polar contacts, within the acceptable range of 2.5-3.5 Å for hydrogen bonding, between residues of both proteins and are shown as dashed yellow lines and by the yellow star. Panel D: Cartoon model depicting the interaction between the RBD of the hIP (residues 299-312) with the α4 helix domain of Rab11a (residues 136-145), where the structure of the RBD is equivalent/derived from that proposed for the active state of the hIP. PyMOL was used to identify potential polar contacts, within the acceptable range of 2.5-3.5 Å for hydrogen bonding, between residues of both proteins and are shown as dashed yellow lines and by the yellow stars. Panel E: Surface representation of the inactive (green) and active (pink) hIP in complex with Rab11a, where the inactive and active homology models of the hIP have been superimposed by structural alignment using PyMOL. Homology modelling predicts that the contact points of Rab11a depend on the activation state of the hIP. More specifically, it is proposed that in going between the inactive to the active state of the hIP, there is a combination of structural changes within the α-H8 domain of the hIP, in addition to deacylation of residues at Cys308 and Cys309 [6]. Such changes, in turn, result in an altered interaction of the hIP with Rab11a to enhance the contacts of Glu138 and Lys145 within the α4 helix of Rab11a with key residues (Lys304, Cys309 and Leu310) within the RBD of the hIP. In the models presented, including in Panel E, the hIP and Rab11a in the inactive hIP:Rab11a complex are depicted in green and blue, respectively, and in the active hIP:Rab11a complex are depicted in pink and orange, respectively.
Figure 6: Effect of Glu138:Lys304 in the constitutive and agonist-induced Rab11a:hIP interactions

Panels A & C: HEK 293 cells, transiently co-transfected with pHM6 plasmids encoding HA-tagged forms of the hIP, hIP<sub>K304A</sub>, hIP<sub>K304R</sub> or hIP<sub>K304Q</sub> in the presence of pEGFP-C1 encoding either GFP-tagged Rab11a (Panel A) or Rab11a<sup>E138A</sup> (Panel C), were incubated with vehicle (0) or 1 μM cicaprost for 2 hr prior to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE, and subject to successive immunoblotting (IB) versus anti-GFP antibody (upper panels) or anti-HA 3F10 HRP-conjugated antibody (middle panels). To verify expression of the Rab proteins, aliquots of whole cell lysate (50 μg/lane) were immunoblotted with anti-GFP antibody (lower panels). The relative positions of the molecular size markers (kDa) are indicated to the left of the panels. Panels B & D: The bar charts show the mean agonist-induced fold increase in the levels of the respective Rab11a/ Rab11a<sup>E138A</sup> proteins associated with the immunoprecipitates of the hIP (wild type/WT, hIP<sup>K304A</sup>, hIP<sup>K304R</sup> or hIP<sup>K304Q</sup>) as determined by quantitative densitometry (± SEM, n ≥ 3). The asterisk symbols (****) indicates where cicaprost stimulation resulted in a significant increase in the agonist-induced interaction between the hIP and Rab11a, where *** indicates p < 0.001 (t test). The hash symbols (#) indicate where mutation of K<sup>304</sup> within the hIP resulted in a significant change in the constitutive interaction between the hIP and Rab11a compared to the wild-type hIP, where ## and ### indicate p < 0.01 and p < 0.001, respectively (Dunnett’s multiple comparison post hoc test following one-way ANOVA).
Figure 7: The α4 helix of Rab11a is a novel binding site within Rab GTPases

Panel A: Surface representation of selected crystal structures of Rab proteins (blue) in complex with their established effector or other regulatory proteins (yellow), where the Rab α4 helix is shown in red and the Switch I/II regions are highlighted in dark grey. FIP2: Rab11-FIP2 in complex with Rab11a (PDB# 2GZD, [32]), RabGAP: RabGAP domain of Gyp1TBC in complex with Rab33 (PDB# 2G77, [61]), RabGDI: Yeast RabGDI
in complex with Ypt31 (PDB# 3CPJ, [60]), RabGEF: RabGEF domain of DENND1B in complex with Rab35 (PDB# 3TW8, [63]), REP: REP-1 in complex with mono-isoprenylated Rab7 (PDB# 1VG0, [62]). Within each of the complexes that have been characterised to date, the interaction between the Rab protein and its binding partner is predominantly mediated through the Switch I and Switch II domains of the Rab GTPase. Panels B & C: Surface representations (Panel B) and schematic models (Panel C) of (i) the proposed complexes of the inactive hIP (green) and Rab11a (blue) and, (ii) the proposed complex of the active hIP (pink) and Rab11a (orange). In each panel, the Rab11-binding domain (RBD) of the hIP, comprising α-H8 and adjacent Cys308-311 residues, is highlighted in white, the α4 helix of Rab11a (residues 136-145) is shown in red and the Rab11a Switch I/II regions are highlighted in dark grey. In the absence of agonist (i), the inactive hIP constitutively binds Rab11a through an interaction mediated, at least in part, through its RBD. Molecular modelling of the inactive hIP-Rab11a structure depicts Lys304 of the RBD and Glu138 of the Rab11a α4 helix forming a contact within the complex. Mutation of these residues shows that while Glu138 is not explicitly required to stabilise interaction of Rab11a with the inactive hIP, the presence of a Lys at residue 304 within the inactive hIP is a key determinant stabilising the complex. These findings suggest that Glu138 on Rab11a may not be the sole determining residue required for interaction with the inactive hIP and that contacts between other residues within the Rab11a α4 helix, or elsewhere within the Rab11a protein, may be sufficient to mediate stable complex formation in the absence of agonist. Upon stimulation with agonist (ii), the active hIP undergoes significant conformational changes relative to the inactive state model. Furthermore, agonist-regulated deacylation of palmitoylated residues within the RBD, and in particular at Cys309, may lead to altered flexibility and positioning of the RBD. Molecular modelling of the active hIP-Rab11a structure predicts that Lys304 of the RBD and Glu138 of the Rab11a α4 helix again form a contact within the active complex. Stabilisation of the complex is potentially enhanced through additional contacts predicted between residues of the Rab11a α4 helix and Cys309 and Leu310 of the active hIP. Disruption of the key Lys304-Glu138 contact in the active hIP-Rab11a complex shows that Glu138 is critically required to stabilise interaction of Rab11a with the active hIP.
Mulvaney et al., Identification of α-helix 4 (α4) of Rab11a as a novel Rab11-binding domain (RBD): Interaction of Rab11a with the Prostacyclin Receptor. (2017), BBA(MCR) 1864, pages 1819 – 1832.

SUPPLEMENTAL DATA:

Supplemental Table 1: PCR Amplification Primers

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</table>

* Plasmids were generated by subcloning the respective PCR-amplified Rab11a subfragments from pEGFP-C1:Rab11a into the 5’ Ncol and 3’ EcoRI sites of pACT2, in-frame with the translation of the activation domain (AD) of the yeast GAL4 transcriptional activator (GAL4-AD). All plasmids were validated by DNA sequence analysis (Eurofins MWG Operon; Ebersberg, Germany).

** Oligonucleotide sequences are shown 5’→3’. Cloning sites are underlined in italics.
### Supplemental Table 2: Site-directed Mutagenesis Primers

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<td>pEGFP-C1:Rab11a&lt;sup&gt;E138A&lt;/sup&gt; (ii)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
</tr>
<tr>
<td>pET-28b:Rab11a&lt;sup&gt;147-173, E138A, E144A&lt;/sup&gt; (iv)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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<tr>
<td>pACT2:Rab11a&lt;sup&gt;124-216, R140A&lt;/sup&gt; (i)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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<tr>
<td>pEGFP-C1:Rab11a&lt;sup&gt;R140A&lt;/sup&gt; (ii)</td>
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</tr>
<tr>
<td>pET-28b:Rab11a&lt;sup&gt;147-173, R140A&lt;/sup&gt; (iii)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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<tr>
<td>pACT2:Rab11a&lt;sup&gt;124-216, F142A&lt;/sup&gt; (i)</td>
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<tr>
<td>pEGFP-C1:Rab11a&lt;sup&gt;F142A&lt;/sup&gt; (ii)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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<tr>
<td>pET-28b:Rab11a&lt;sup&gt;147-173, F142A&lt;/sup&gt; (iii)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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<tr>
<td>pACT2:Rab11a&lt;sup&gt;124-216, K145A&lt;/sup&gt; (i)</td>
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<td>pEGFP-C1:Rab11a&lt;sup&gt;K145A&lt;/sup&gt; (ii)</td>
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<tr>
<td>pET-28b:Rab11a&lt;sup&gt;147-173, K145A&lt;/sup&gt; (iii)</td>
<td>GCCAGTTCTACAGAATCAGCAAGAGCTTTTGCAAG</td>
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</table>

* Plasmids were generated by QuikChange site-directed mutagenesis using either (i) pACT2:Rab11a<sup>124-210</sup>, (ii) pEGFP-C1:Rab11a, (iii) pET-28b:Rab11a<sup>147-173</sup> or (iv) pET-28b:Rab11a<sup>147-173, E144A</sup> as template. All plasmids were validated by DNA sequence analysis (Eurofins MWG Operon; Germany).

** Oligonucleotide sequences are shown 5’ → 3’ and correspond to those of the sense primer only; the antisense sequence is inferred. The identity of the mutator codon is in boldface italics with the actual mutated base(s) underlined.
Supplemental Figure 1: Y2H Control Screens

*S. cerevisiae* Y187 (pTD1-1), encoding the SV-40 large T-antigen, and *S. cerevisiae* Y187 (pACT2) prey strains were mated with *S. cerevisiae* AH109 bait strains transformed with recombinant pGBK7 encoding the listed subfragments of the hIP and, as controls, *S. cerevisiae* AH109 (pGBK7:p53) or *S. cerevisiae* AH109 (pGBK7) (Ø). Diploids were selected by growth on DDO medium, whereas interactants were selected on QDO medium, and by their ability to express β-Gal, as a result of GAL4-dependent transcriptional activation of the HIS3, ADE2 and lacZ reporter genes, respectively, due to interaction between bait and prey proteins. Data presented are representative of at least three independent experiments (n ≥ 3).
Supplemental Figure 2: Interaction of Rab11a with the hIP

*S. cerevisiae* Y187 (pACT2:Rab11a<sub>124-216</sub>), or *S. cerevisiae* Y187 strains harbouring the listed carboxyl-terminal truncated deletions of Rab11a<sub>124-216</sub>, were mated with *S. cerevisiae* AH109 bait strains transformed with recombinant pGBK7 encoding the listed subfragments of the hIP and, as controls, *S. cerevisiae* AH109 (pGBK7:p53) or *S. cerevisiae* AH109 (pGBK7) (Ø). Diploids were selected by growth on DDO medium, whereas interactants were selected on QDO medium, and by their ability to express β-Gal. Data presented are representative of at least three independent experiments (n ≥ 3).
Supplemental Figure 3: Effect of Ala-scanning mutagenesis of the α4 helix of Rab11a on its interaction with the hIP

HEK.hIP^WT cells were transiently transfected with pEGFP-C1 encoding GFP-tagged Rab11a, or the listed Ala-scanning variants of the α4 helix region of Rab11a, were incubated with vehicle (0) or 1 μM cicaprost for 2 hr, prior to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE, and subject to successive immunoblotting (IB) versus anti-GFP antibody (upper panels) or anti-HA 3F10 HRP-conjugated antibody (middle panels). To verify expression of Rab11a and of its Ala-scanning variant proteins, aliquots of whole cell lysate (50 μg/lane) were immunoblotted versus anti-GFP antibody (lower panels). The relative positions of the molecular size markers (kDa) are indicated to the left of the panels.
Supplemental Figure 4: Biophysical characterisation of the interaction of Rab11a with the hIP

Representative SPR sensorgrams from a binding screen of the purified wild-type Rab11a (WT), the individual E138A (Rab11a\textsuperscript{E138A}), individual E144A (Rab11a\textsuperscript{E144A}), or the dual E138A & E144A (Rab11a\textsuperscript{E138A,E144A}) Ala-scanning variant proteins screened vs the immobilized hIP RBD peptide (residues 296-312; pepIP\textsuperscript{296-312}). Rab11a proteins were injected at 1 μM for 2 min over a Series S streptavidin-coated (SA) sensor chip with immobilized pepIP\textsuperscript{296-312} followed by buffer flow for 3 min.
Supplemental Figure 5: Expression and Characterisation of Rab11a Fusion Proteins

Panel A: SDS-PAGE analysis of the purification of Rab11a WT and the listed Ala-scanning variants of the α4 helix region of Rab11a, respectively, where 0.5 μg of each protein were loaded per lane and subsequently stained with Coomassie Brilliant Blue. Panel B: Far-UV CD spectra of Rab11a WT and the listed Ala-scanning variants of the α4 helix region of Rab11a. All spectra indicate similar secondary structure to that of the WT protein. Scans were carried out in 10 mM Tris, 10 mM NaCl, 1 mM DTT pH8.0 at 25 °C, and spectra were converted to Mean Residue Ellipticity (MRE) [θ] values. Spectra are coloured according to the legend.